VIII. THE RELATION OF PYRUVIC ACID IN BRAIN TO CERTAIN TISSUE POISONS.

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(*Received November 3rd, 1934.*)

Some indication was obtained by Peters and Thompson [1934] that fluoride diminished the substantial amount of pyruvate formed by avitaminous brain, as well as the smaller amounts formed in normal brain respiration. On the other hand sodium iodoacetate led to increase of pyruvate even in normal brain; consideration of this for avitaminous brain was left until more data became available. This study was undertaken in the hope of deciding the point of action of vitamin B_1 and also of testing further the applicability of the Embden-Meyerhof scheme to brain systems. According to Meyerhof and Kiessling [1933], iodoacetate and fluoride inhibit different stages in the fermentation cycle; fluoride inhibits the appearance of pyruvic acid from phosphoglycerate and iodoacetate the disappearance of the former; the action of each gives rise to inhibition of lactic acid formation.

Oxygen uptake.

Table I (*a*) summarises the experiments which we have made upon respiration in the presence of the two poisons. The results are given as averages. It is to be understood that in each series of experiments the control and poison have been tested in duplicate upon the same brain. The results for fluoride confirm and extend those of Peters and Sinclair [1933]; the concentration used ($0{\cdot}024\,M$) is taken from their work. With iodoacetic acid the most suitable concentration was found to be $0{\cdot}00054\,M$ (0·1 mg. per ml.); this is the amount found to inhibit the action of glyoxalase in tissue sections [Dickens, 1933].

In orientating experiments upon normal brain, $0{\cdot}0009\,M$ seemed maximum, $0{\cdot}00018\,M$ gave less effect. With avitaminous brain in four experiments, $0{\cdot}00018$–$0{\cdot}00027\,M$ gave approximately the same effect, $0{\cdot}000095\,M$ gave sometimes less.

The following can be concluded from Table I (*a*). Both poisons reduce the oxygen uptake in lactate solutions; the percentage of respiration so inhibited increases during the period studied, but the absolute amounts inhibited in μl./g./hr. remain surprisingly constant, if we exclude the first period. For avitaminous brain the inhibition amounts to 500 μl./g./hr. in every case except that of fluoride and pyruvate, where the average was 150. This curious difference with pyruvate is probably related to the observation of Meiklejohn *et al.* [1932] that there is a tendency for respiration of avitaminous brain to be lower in pyruvate solutions. The effect is not constant, because in two further experiments carried out recently by the latest technique, in which the actions of fluoride and iodoacetic acid with pyruvate were tried on the same brain, one showed the difference and the other did not (Exps. 692, 693, Table I (*b*)). Some unknown variation in the state of the brains must influence the result. With this exception, the effects of the two poisons upon respiration are identical.

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Table I. *Effect of poisons upon respiration of avitaminous brains.*

(*a*) *Effects of fluoride and iodoacetate upon oxygen uptake.* μl./g./hr.

Average respiration rates. January-July.

	No. of exps.	Substrate	Poison	Period (hrs.) 0–½	½–1	1–1½	1½–2
Normal	5	Lactate	—	2600	2210	1880	1580
		,,	Fluoride	1640	1230	810	550
	3	Lactate	—	2715	2305	1965	1690
		,,	IAA	1923	1013	533	275
Avit.	3	Lactate	—	1650	1280	980	800
		,,	Fluoride	1070	725	465	305
	3	Lactate	—	1730	1295	985	840
		,,	IAA	1285	755	435	280
	4	Pyruvate	—	1085	790	600	465
		,,	Fluoride	660	600	405	305
	4	Pyruvate	—	1465	1035	775	585
		,,	IAA	1035	535	320	185
	10	Lactate + pyruvate	—	1450	1020	800	650
			IAA	1100	595	340	230

Summary of differences. μl./g./hr.

	Substrate	Poison	0–½	½–1	1–1½	1½–2	% resp. stable to poison 0–½	½–1	1–1½	1½–2
Normal	Lactate	Fluroide	960	980	1070	1030	63	56	43	38
	,,	IAA	790	1290	1430	1420	71	44	27	16
Avit.	Lactate	Fluoride	580	555	515	495	65	57	47	38
	,,	IAA	445	540	550	560	74	58	44	33
	Pyruvate	Fluoride	425	190	195	160	68	76	68	66
	,,	IAA	430	500	455	400	71	52	42	32
	Lactate + pyruvate	IAA	350	425	460	420	76	59	43	35

(*b*) *Pyruvate—fluoride—IAA. September.*

Avit. Exp.	Substrate	Poison	0–½	½–1	1–1½	1½–2	Difference 1–1½
692	Lactate	—	2180	1835	1330	1000	
	Pyruvate	—	1710	1350	1030	830	
	,,	Fluoride	1125	850	530	350	500
	,,	IAA	1180	580	340	160	690
693	Lactate	—	1850	1280	925	720	
	Pyruvate	—	2140	1595	1370	1105	
	,,	Fluoride	1270	820	470	335	900
	,,	IAA	1595	850	475	280	895

(*c*) *Catatorulin effect—average values.*

Increase produced by vitamin in poisoned systems.

No. of exps.	Substrate	Value	0–½	½–1	1–1½	1½–2
2	Lactate	(L + F + V) − (L + F)	160	90	115	60 μl.
4	Pyruvate	(P + F + V) − (P + F)	160	200	140	140 μl.
10	Lactate − pyruvate	(LP + IAA + V) − (LP + IAA)	195	100	80	60 μl.
	% total vitamin effect, *i.e.* of (LP + V) − LP		46	20	15	10

Table I (*cont.*).

(*d*) *Influence of fluoride + IAA upon catatorulin effect.*

Exp.						
612	LP	1700	1365	1020	880	
	LPV	1980	1805	1240	1360	
	LPIAA	1700	980	380	170	
	LPIAAV	1840	1165	655	470	
	LPIAAF	1115	555	310	205	
	LPIAAVF	1255	720	320	245	
613	LPIAA	825	470	305	200	
	LPIAAV	1090	815	585	428	IAA. 0·12 mg./ml.
	LPIAAF	565	365	210	136	
	LPIAAVF	630	390	235	185	

In this and other Tables L = lactate; P = pyruvate; IAA = iodoacetic acid; F = fluoride; V = vitamin B_1.

Note. It is to be understood that in all cases medium consisted of Ringer phosphate-pyrophosphate with addition of lactate 0·03 *M* or pyruvate 0·02 *M*. LP = L 0·03 *M*, P 0·01 *M*.

The catatorulin effect (Table I (*c*)) is present but reduced to somewhat the same extent in each case; Exps. 612, 613 (Table I (*d*)) show that the poisons reinforce each other's action; both together eliminate the vitamin action.

Effect upon preformed pyruvate.

Avitaminous brain was allowed to form pyruvate by respiration for 2 hours in oxygen and lactate solution, at the end of which time additions of vitamin and tissue poisons were made. Pyruvate was estimated in most cases as bisulphite-binding substances [Clift and Cook, 1932] at the end of a further 1¼ hours. This was occasionally checked by Case's method.

The details of the experiment were as follows. Avitaminous cerebrum, minced warm, was distributed in five bottles containing Ringer phosphate and lactate 0·03 *M*. Pyruvate was formed by allowing respiration in O_2 for 2 hours from time of putting in the bath, the oxygen uptake being recorded for the last half hour, in order to check the values. At the end of this time, one bottle was removed as control and treated with 0·9 ml. trichloroacetic acid (25 %). The remaining four bottles were removed from the bath, one kept as control, one treated with vitamin (0·1 ml. containing 2γ), another the poison in 0·2 ml. and the last poison and vitamin; the bottles were quickly re-evacuated, filled with oxygen and returned to the bath. This operation need not occupy more than 7 minutes. Respiration was allowed to continue for 1 hr. 12 mins. after returning to the bath, the rate being calculated for the periods 12–42 mins. and 42–72 mins. No pyrophosphate was used in order to minimise the effects of the preparative stage (see preceding paper). At the end of 42 mins. the remaining bottles were removed from the bath and treated with trichloroacetic acid. Other details were as described by Peters and Thompson [1934].

It was thought better in this experiment to depart from the usual practice of using duplicate bottles for each point, and to use instead larger amounts of tissue from the cerebrum only, which is well known to be more homogeneous. Attempts to weigh exact amounts of brain "brei" introduce in themselves the qualitative errors of uneven cooling during the interval required for such weighing. We have therefore followed the usual practice of distributing the tissue by eye. This has not always been successful. We have therefore corrected for the tissue weights in the following way.

Variations in tissue weight for amounts of tissue over 100 mg. were found by Meiklejohn not to affect the oxygen uptake over 3-hour periods in the case of the cerebrum. This is contrary to the calculations of Passmore *et al.* [1933], who were able to correct for small amounts of tissue by the application of a formula. The difference between these two sets of results is not yet clear. The following diagram (Fig. 1) shows that oxygen uptake is independent of the amount of tissue for amounts between 140 and 250 mg. but the amount of pyruvic acid varies with tissue weight.

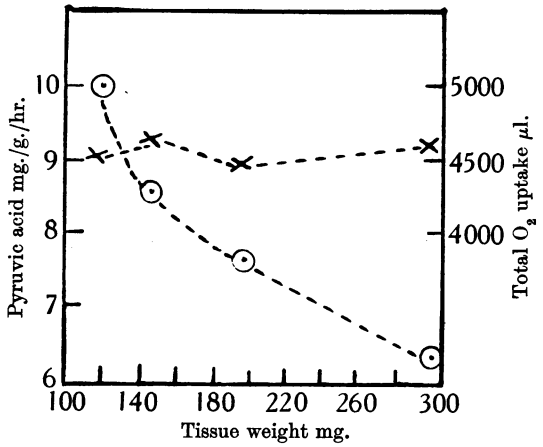

Fig. 1. ⊙ Pyruvic acid formed per tissue wt. × O_2 uptake per tissue wt.

Table II. *Pyruvic acid, poisons and the catatorulin effect. Typical experiments.*

Oxygen uptake. ↓, substrate addition.

Exp. 678.

Hours ...	(*a*) 1½–2	↓	(*b*) 2¼–2¾	(*c*) 2¾–3¼
L	1195		815	600
LF	1180		710	420
LV	1110		990	1000
LFV	1120		880	650

Exp. 683.

Hours ...	(*a*) 1½–2	↓	(*b*) 2¼–2¾	(*c*) 2¾–3¼
L	710		590	275
LIAA	700		475	355
LV	700		700	580
LIAAV	700		625	415

Average decrease in respiration for (*b*) and (*c*) as compared with (*a*).

	(*b*) %	(*c*) %
L	−24	−50
LV	− 9	−19
LF	−42	−66
LFV	−21	−46

	(*b*) %	(*c*) %
L	−27	−55
LV	−10	−19
LIAA	−37	−55
LFV	−20	−33

Pyruvic acid. Pyruvate estimations corrected to weight of 160 mg. tissue weight along tangent to curve.

ml. *N*/100 I/g. (1 ml. = 0·44 mg. pyruvic acid).

Fluoride:

Exp.	Initial L	Final L	L+F	L+V	L+V+F
678	**6·19**	**9·8**	8·6	**7·2**	**7·44**
679	5·7	**8·3**	**6·2**	6·8	**5·7**
680	5·7	**7·9**	**6·9**	**4·9**	5·4
685	—	**9·7**	**8·4**	**8·0**	7·6
686	—	9·7	**8·5**	7·2	**7·2**
Average	5·9	9·1	7·7	6·0	6·7 ml. I/g.

Iodoacetate:

Exp.	Initial L	Final L	L+IAA.	L+V	L+V+IAA.
681	5·9	7·1	**8·1**	**6·0**	**7·3**
682	**9·0**	13·0	**11·3**	7·8	9·8
683	**7·4**	**9·2**	**8·9**	7·9	8·9
684	5·7	**7·7**	**8·1**	5·7	7·0
687	**7·3**	8·8	9·7	7·1	**8·5**
Average	7·1	9·2	9·2	6·9	8·3

Note. In 682 and 683, L+V, L+V+IAA, also same wt.

This is incidental evidence of the lack of correlation between O_2 uptake and pyruvic acid formation. The results given in Table II have been corrected for initial pyruvate (at end of 2 hours) to a basis of 160 mg. using the curve given in the figure. The conclusions which emerge from the averages are not however dependent upon this; in several cases tissue weights have been sufficiently close to require no correction. These are marked in heavy type.

Table II confirms the finding of Table I, that the O_2 uptake is reduced equally within the wide limits of error by the two poisons. The effect upon the pyruvate is strikingly different. It is decreased by fluoride and not affected by iodoacetate. In confirmation of Peters and Thompson, vitamin reduces the amount of pyruvate present. We must suppose that removal of pyruvic acid is already at a minimum in the avitaminous tissue in absence of vitamin so that no further accumulation takes place in presence of iodoacetate. So far as the origin and disappearance of pyruvic acid are concerned, the results are in good agreement with Meyerhof and Kiessling [1933]. As with the oxygen uptake, they do not give ground for the belief that the action of the vitamin is more concerned with iodoacetate than with fluoride, since no stress can be laid upon the variable results with pyruvate and fluoride. It must be noticed that in presence of vitamin, fluoride does not decrease significantly the amount of pyruvate formed.

Glutathione and added pyruvate.

Relatively more is known about the action of iodoacetic acid than about that of fluoride. We have therefore tried to analyse this further. The following is selected from the literature as to the action of iodoacetic acid.

1. Inhibition of lactic acid formation in muscle [Lundsgaard, 1930] and other tissues [Fisher, 1931].

2. Reduction of tissue respiration in muscle, which is partly restored by lactate addition [Meyerhof and Boyland, 1931]. Lactate improves contraction of muscle supplied with oxygen and poisoned with iodoacetate, and phosphagen synthesis [Mawson, 1932; 1933]; rather similar effects occur with nerve [Chang and Gerard, 1933].

3. Low concentrations interfere with the catabolism of glycogen (A), Meyerhof and Kiessling [1933], and with the action of glyoxalase in tissue sections (B) [Dickens, 1933; Quastel and Wheatley, 1932. *Cf.* Dudley, 1931].

4. Higher concentrations prevent the interaction of α-glycerophosphate and pyruvic acid (C). They also inhibit the action of glyoxalase in some brei preparations, the inhibition being believed to be due to the removal of glutathione [Dickens, 1933].

Hence at least three enzyme systems A, B, C, must be poisoned. Our present effect might be due to B or C. If to B alone it should be restored by adding glutathione.

In a series of seven experiments the effect of glutathione with and without vitamin upon pyruvate was studied in presence of iodoacetate. In order to ensure the presence of adequate pyruvate, a small amount of this was added to the medium, which also contained pyrophosphate to enhance vitamin effects. Some of the O_2 uptakes are summarised in Table I. In Table III, 1, 2, 3 are given only the figures relating to glutathione and the values for pyruvate.

In order to observe the action properly, the tissue should be kept in contact with the reagents for a standard time before adding the glutathione. In practice the tissue was allowed to stand for 3 minutes after division with the crusher, iodoacetate was then added, after an interval of 2 minutes glutathione and after a further interval vitamin.

Table III. *IAA, glutathione and pyruvate.*

Medium (M) = lactate-pyruvate-pyrophosphate. G = glutathione, 0·0021 *M*. V = vitamin 2γ.

1. *Oxygen uptake.* Average level for 7 experiments.

		Period (hours)			
		0–½	½–1	1–1½	1½–2
M.IAA		1100	595	340	230
M.G.IAA		1200	760	570	390
	Diff.	100	165	230	160
M.IAA.V		1295	695	420	290
M.IAA.V.G		1450	1080	795	620
	Diff.	155	385	375	330

2. Effect of glutathione and iodoacetic acid on the removal of added pyruvic acid: expressed as mg. pyruvic acid disappearing per g. of tissue.

Exp.	M	MV	MIAA	MIAAV	MIAAG	MIAAGV
600	−0·56	−2·73	−0·22	−1·63	−0·41	−1·97
601	+0·09	−3·14	+1·03	−2·92	−0·21	−1·91
604	−0·78	−4·66	+1·00	−0·10	+0·32	+2·77
605	−2·24	−4·41	−0·53	−1·12	−1·62	−4·16
607	−1·09	−2·51	+0·79	+0·13	−0·38	−2·43
608	−0·97	−3·14	−0·10	−1·43	—	—
612	−2·70	−4·47	−0·35	−1·47	—	—

3. Effect of glutathione and iodoacetic acid on the vitamin effect.

In 3, M≡MV − M, etc.

(A) Extra disappearance of pyruvic acid due to vitamin action.

Exp.	M	MIAA	MIAAG
600	2·17	1·41	1·56
601	3·23	3·95	1·70
604	3·87	1·10	3·09
605	2·17	0·59	2·54
607	1·42	0·66	2·06
Average	2·57	1·54	2·19

(B) Extra O_2 uptake due to vitamin action. (μl./g./hr.)

		Period (hours)			
Exp.	Medium	0–½	½–1	1–1½	1½–2
600	M	320	525	590	845
	MIAA	(440)	137	−181	−47
	MIAAG	205	408	123	248
601	M	325	385	505	520
	MIAA	65	−65	10	35
	MIAAG	220	265	227	190
602	M	545	735	713	705
	MIAA	115	80	−7	72
	MIAAG	340	340	205	240
603	M	85	345	410	465
	MIAA	(390)	−25	−102	15
	MIAAG	405	475	350	365
604	M	465	400	705	690
	MIAA	−295	105	54	83
	MIAAG	135	100	97	45
605	M	555	640	615	805
	MIAA	260	180	199	55
	MIAAG	325	400	415	422
607	M	335	325	490	495
	MIAA	90	−55	180	−25
	MIAAG	130	200	230	136
Averages	M	375	479	575	646
,,	MIAA	47	51	22	27
,,	MIAAG	251	318	235	235
Actual restoration with G (MIAAG–MIAA)		204	267	213	208
% restoration with G		54·5	56	37	32

Note. In Exp. 600, the pyruvic acid was estimated by the isolation of the 2 : 4-dinitrophenylhydrazone, in all other experiments by bisulphite-iodine titrations.

The increases seen in MIAA 601, 604 and 607 are probably not experimental errors, but due to the formation of pyruvate by the tissue (compare Table II).

In these experiments addition of glutathione restored the vitamin effect to some 30–50 %. Part too of the pyruvate seems to be removed again upon addition of glutathione, but this cannot be certainly concluded owing to the large experimental error. The dubiety of this result makes interpretation difficult. Iodoacetate is known to interact slowly, and it is not known how far glutathione will diffuse into these tissue systems, though in general it is freely diffusible. We think it probable that the glutathione is merely combining with the excess of iodoacetate, thereby preventing further action; if the effect were to restore a missing catalytic effect of glutathione we should have expected the recovery with the added substance to be more nearly maximum. Hence we do not think that glutathione is directly concerned with the vitamin effect; in this we have the support of earlier experiments with glutathione alone. So far judgment from the respiration experiments of Peters and Sinclair [1933] excludes interaction with α-glycerophosphate. Hence both A and C seem also excluded and we are left with the inhibition of some unknown metabolic link.

Table IV. *Delayed substrate experiments with poisons.*

Each experiment made upon uniform sample of brain tissue.

Exp. No.	Initial addition	Addition at ↓	$1\frac{3}{4}$–$2\frac{1}{4}$		Resp. period from start of experiment (hours) $2\frac{1}{2}$–$2\frac{3}{4}$	$2\frac{3}{4}$–$3\frac{1}{4}$	$3\frac{1}{4}$–$3\frac{3}{4}$	$3\frac{3}{4}$–$4\frac{1}{4}$	$4\frac{1}{4}$–$4\frac{3}{4}$
Fluoride:									
641	O	LPppV	420	↓	450	390	350	330	—
	O	O	470	↓	310	230	230	170	—
	V	LPpp	545	↓	520	625	600	535	—
	V	O	640	↓	455	440	340	280	—
	V	LPppF	590	↓	580	520	340	280	—
	VF	LPpp	220	↓	300	175	130	90	—
645	V	LF	700	↓	855	885	845	800	—
	O	LFV	500	↓	570	575	625	615	—
	F	LV	190	↓	200	160	160	120	—
	F	V	170	↓	90	60	150	70	—
	FV	L	240	↓	270	220	170	174	—
	FV	O	260	↓	230	120	150	100	—
646	O	PFV	550	↓	445	435	290	190	—
	V	PF	625	↓	680	740	480	270	—
	F	PV	190	↓	195	210	185	65	—
	F	V	215	↓	240	160	85	145	—
	FV	P	225	↓	250	190	215	70	—
	FV	O	220	↓	210	160	150	65	—
IAA:									
642	ppV	LP	660	↓	1170	995	930	935	—
	ppV	O	710	↓	530	495	410	310	—
	pp	LPV	500	↓	770	640	540	635	—
	pp	O	500	↓	480	355	310	290	—
	ppV	LPIAA	575	↓	935	725	460	340	—
	ppV	IAA	605	↓	485	340	250	215	—
	ppIAA	LP	90	↓	300	100	115	85	—
	ppVIAA	O	115	↓	110	40	75	85	—
708	pp	LV	570	↓	870	700	730	780	655
	ppV	L	760	↓	1250	1090	1105	1035	—
	ppIAA	LV	225	↓	325	170	95	135	25
	ppV	L	285	↓	275	230	165	125	90
	pp	LVIAA	625	↓	940	800	640	495	320
	ppV	LIAA	760	↓	1325	1135	800	580	385

O = Ringer-phosphate; V = O + vitamin; pp = pyrophosphate. Pyruvate (P) approx. in Exps. 646, 0·02 *M*; in Exps. 641, 642, 0·007 *M*.

Delayed substrate experiments (Table IV).

There is a component of the preparative stage blocked by the action of the poisons. In experiments made by the delayed substrate technique (see preceding paper), poison in the preparative stage inhibits subsequent rise of oxygen uptake upon addition of lactate. The vitamin effect is eliminated. Poison added at the same time as substrate only acts slowly and does not inhibit the initial rise. We interpret this to mean that the poison acts upon the conversion of X into Y, but not the interaction of Y with substrate[1]. The later reduction of respiration of the tissue treated with lactate and poison at the same time is consistent with this view; it would be expected that after respiration for an hour with substrate, there would be a component of the respiration supplied by further formation of Y. The application of these results to the method of Quastel and Wheatley suggests that narcotics as well as iodoacetic acid will probably be acting upon the preparative stage.

It is to be noted that even after 2 hours of falling residual respiration, the capacity for forming pyruvic acid is not substantially diminished. Table V shows the few experiments made upon this question with avitaminous cerebrum. They suffice to indicate the rapid increase of pyruvate upon addition of lactate and again show the reduction in presence of vitamin. It will be recalled that there are reasons for thinking that pyruvate does not arise directly from lactate [Peters and Thompson, 1934].

Table V. *Formation of pyruvate in delayed substrate experiment.*

Bisulphite-binding substances present after respiration for periods indicated. ml. N/100 I/g. tissue (avitaminous cerebrum).

Exp.	RP After 2 hrs.		RP Hours +¼	RP Hours +¾	L Hours +¼	L Hours +¾	L +1 hr. 10 mins.	LV Hours +¼	LV Hours +¾	LV +1 hr. 10 mins.
671	—	↓	2·6	2·2	3·5	4·5	—	—	—	—
673	—	↓	—	—	3·4	4·0	—	2·5	2·6	—
675	1·6	↓	—	—	3·6	—	5·5	3·3	—	3·3
676	2·3	↓	—	—	3·8	5·4	—	3·8	3·6	—
Average	2·0	↓	2·6	2·2	3·6	4·6	—	3·2	3·1	—

RP = Ringer phosphate only. At arrow (end of 2 hours) addition of L or LV or nil made as indicated and respiration continued for additional periods.

Discussion.

Catatorulin effect. The catatorulin effect is inhibited by action upon the preparatory stages equally by the two poisons, irrespective of the effect upon pyruvate. This reinforces the view that the action of vitamin upon pyruvate is secondary. It seems to be a generalisation that poisons influencing catatorulin action affect phosphorylation stages. This is even true for phloridzin [Lundsgaard, 1933]. With 3 mg./ml. in three experiments the "vitamin effect" was almost eliminated. There is no evidence that any of these poisons is specific for vitamin B_1. Iodoacetate destroys vitamin B_1 activity upon warming at p_H 7·3 in Ringer phosphate for 12 mins., but a short period of standing at room temperature hardly influences it.

Embden-Meyerhof scheme. This is followed to the extent that fluoride and iodoacetate influence appearance and disappearance of pyruvate as in the

[1] See hypothesis in the previous paper.

scheme. According to Johnson (in press)[1], the scheme cannot be applied so far as α-glycerophosphate is concerned.

Lactic acid. Chang and Gerard [1933] have found that with lactate present the fall in respiration of frog nerve is not so marked at first as subsequently in iodoacetate solutions. The figures of Table I bear this out for pigeon's brain. It is well explained by the hypothesis here advanced if iodoacetate poisons production of *Y*.

Summary.

1. The actions of some poisons upon the avitaminous pigeon's brain tissue have been further investigated. Fluoride and iodoacetate reduce O_2 uptake equally. Each largely reduces the catatorulin effect (extra O_2 uptake in presence of added vitamin B_1): both together eliminate this.

2. The effect upon pyruvate formation by avitaminous brain is different; iodoacetate leads to increased accumulation and fluoride the reverse. So far only as pyruvate is concerned, this is consistent with the finding of Meyerhof and Kiessling for muscle extracts.

3. Glutathione only produces partial restoration of the vitamin effect in presence of iodoacetate.

4. Disappearance of pyruvate in presence of vitamin B_1 is an indirect result of some other change. These poisons influence a preparatory stage for oxidation.

[1] Personal communication.

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